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Stem cells: attributes, cycles, spirals, pitfalls and uncertainties

Lessons for and from the Crypt

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(A) Summary

We consider some of the problems involved in current discussions on stem cells in adult mammalian tissues. The present concepts involve a number of pitfalls, weaknesses and logical, semantic and classification problems. This indicates the necessity for new and well-defined concepts that are amenable to experimental analysis.

One of the major difficulties in considering stem cells is that they are defined in terms of their functional capabilities which can only be assessed by testing the abilities of the cells, which itself may alter their characteristics during the assay procedure: a situation similar to the uncertainty principle in physics. The terms that describe stem cell functions are often not well defined and are used loosely, which can lead to confusion.

If such context-dependent interactions exist between the manipulation and measurement process and the challenged stem cells, the question of, for example, the number of stem cells, in a tissue has to be posed in a new way. Rather than obtaining a single number one might end up with various different numbers under different circumstances, all being complementary. This might suggest that stemness is not a property but a spectrum of capabilities from which to choose. This concept might facilitate a reconciliation between the different and sometimes opposing experimental results.

Given certain experimental evidence, we have attempted to provide a novel concept to describe structured cell populations in tissues involving stem cells, transit cells and mature cells. It is based on the primary assumption that the proliferation and differentiation/maturation processes are in principle independent entities in the sense that each may proceed without necessarily affecting the other.

Stem cells may divide without maturation while cells approaching functional competence may mature but do not divide. In contrast, transit cells divide *and* mature showing intermediate properties between stem cells and mature functional cells. The need to describe this transition process and the variable coupling between proliferation and maturation leads us to formulate a *spiral model of cell and tissue organisation*.

This concept is illustrated for the intestinal epithelium. It is concluded that the small intestinal crypts contain 4–16 *actual stem cells* in steady state but up to 30–40 *potential stem cells* (clonogenic cells) which may take over stem cell properties following perturbations.

This implies that transit cells can under certain circumstances behave like actual stem cells while they undergo maturation under other conditions. There is also evidence that the proliferation and differentiation/maturation processes are subject to controls that ultimately lead to a change in the spiral trajectories. The consequences of the concept in relation to hyperplasia and cancer development are also discussed.

Key words: stem cells, intestinal crypts, cell division, carcinogenesis, biomathematical modelling, differentiation, selfmaintenance, tissue organisation.

(B) Introduction

The stem cells in adult mammalian tissues (and the property of stemness) are difficult to define conceptually, largely impossible to identify morphologically and are associated with functions and attributes that commonly confuse rather than clarify their identity and role. Here, we wish to define the population of stem cells in adult mammalian tissues; to consider the concepts and implications behind such a definition and to apply this definition to one particular system: the epithelium in the small intestinal crypts. In doing so we will identify other terms and concepts that require clarification before a meaningful use of the term 'stem cell' can be made. The application of the concept to a specific tissue, the small intestine, itself results in, as it were, a feed-back loop since we learn and modify our ideas as we apply the term and consider existing, and design new, experiments to investigate the problem. We shall be considering primarily intestinal crypts under conditions where cell production balances cell loss, i.e. *steady state* and also in perturbed situations where there is compensation for injury to the system. Other tissues will be considered where appropriate, as will the developmental sequence in the establishment of stem cell populations since one seeks a general model that will apply in a broader range of situations. However, it should be noted that precise details of the expansion of stem cell numbers and their spatial distribution during development are still poorly understood.

(C) Definitions

(1) *Definition of stem cells*

Stem cells are defined by virtue of their functional attributes. This immediately imposes difficulties since in order to identify whether a cell is a stem cell or not its function has to be tested. This inevitably demands that the cell must be manipulated experimentally, which may actually alter its properties. We will return to this circular problem later. The second problem faced in defining the stem cell population is that adjectives must be used to describe a function and such words themselves may be open to various interpretations. Hence, inevitably, one has to be somewhat pedantic in defining the terms in order to avoid ambiguities and uncertainties.

We would define the *stem cells as undifferentiated cells capable of. (a) proliferation, (b) selfmaintenance, (c) the production of a large number of differentiated, functional progeny, (d) regenerating the tissue after injury, and (e) a flexibility in the use of these options* (see Lajtha, 1967, 1979*a*.*b*,*c* Steel, 1977; Potten and Lajtha, 1982; Potten 1983*a*. Wright and Alison, 1984; Potten and Morris, 1988; Editorial, 1989; Hall and Watt, 1989). Ideally, in order to categorise a cell, or population of cells, as stem cells, all of these criteria should be satisfied; in practice, because of experimental limitations, only some may be satisfied. This is further complicated by the fact that not all of these functions have the same weighting. For example, it would not be sufficient to characterise a stem cell by virtue of its ability to proliferate alone, although this has been done in the past. Cells or populations of cells actually fulfilling all these criteria at a given instance will be called *'actual stem cells'*, while those not expressing their capabilities, though they possess these capabilities, will be termed *'potential stem cells'*. It may be possible for a stem cell to cease proliferation, i.e. become *quiescent*, in which case it is not an actual stem cell but since it can re-enter the cycle it has the potential to be a stem cell. This concept will be dealt with later in this paper. Here it is sufficient to point out that we may have two classes of stem cells: those that actually satisfy at least some of the requirements of the definition, i.e. actual stem cells, and those that may have the potential to do so under special conditions, i.e. potential stem cells. We choose the word actual in preference to the term functional, which has been used previously (Steel, 1977; Cairnie *et al.* 1965; Wright and Alison, 1984), since there is the possibility of confusion between tissue function, and cell function, which are attributes associated with differentiation and maturation, and stem cell function, which is related to proliferation.

Some terms outlined in our stem cell definition have stronger weight than others and hence some of them could be accepted on their own as a means of identifying stem cells: (1) selfmaintenance and the ability to vary selfmaintenance; (2) the ability to produce a large family of differentiated functional cells; (3) the ability to regenerate the tissue or elements of it by producing a large family of differentiated functional progeny following injury; and (4) *undifferentiated* which is a term that requires a precise understanding of what is meant by differentiation, which we will define later. It is a somewhat weak negative parameter for identifying stem cells since it is a relative term that only has meaning in as much as it describes the absence of properties associated with differentiation. In practice this means that the cells can be identified only as not possessing a differentiation marker in comparison with some other cells. This is a fairly specific criteria but, at

its weakest, undifferentiated is used in its morphological sense that the cell has no physical features commonly attributed to specialised chemical or physical functions.

(2) *General definitions and concepts*

In order to understand the full meaning and implications of the definition of stem cells, we need now to consider some subsiduary definitions. One of the most important is associated with differentiation and maturation.

(a) *Differentiation*

Differentiation can be defined as a qualitative change in the cellular phenotype that is the consequence of the onset of synthesis of new gene products, i.e. the non-cyclic (new) changes in gene expression that lead ultimately to functional competence (see Lajtha 1979c). It may be recognised by a change in the morphology of the cell or by the appearance of changes in enzyme activity or protein composition. Since it is a qualitative change, a cell can be said to be differentiated only relative to another cell and during its life a cell may be capable of undergoing several differentiation events. It is commonly identified by the detection of a novel protein; these days usually utilising monoclonal antibodies. The ability to define a cell as differentiated thus clearly depends on the sensitivity of the detection procedures. A few molecules of a novel protein may be detectable, as may the changes in the messenger RNA responsible for these molecules, but ultimately the differentiation event involves a change in the repression/activation of the genome and this may approximate to a quantal phenomenon. According to this definition cells developing from a primitive stage to functional competence may undergo many, even a series of, differentiation events each linked to a novel change in the gene activation pattern. In many circumstances, it may be practically helpful to consider only some primary key (marker) genes as relevant indicators of differentiation particularly if secondary genes are activated subsequently.

(b) *Maturation*

Maturation in contrast can be regarded as a quantitative change in the cellular phenotype or the cellular constituent proteins leading to functional competence (see Lajtha 1979c). Thus the degree of maturation, in principle, could be measured on a quantitative scale e.g. of the weight of a specific protein per cell. A differentiated cell matures with the passage of time to form a functionally competent cell for that particular tissue. Its passage through time and space could in principle be mapped, as new differentiation events occur changing the path of the cell. This relationship is illustrated in Fig. 1.

(c) *Proliferation*

Proliferation is a process involving a sequential pattern of (cyclic, repeating) changes in gene expression leading ultimately to the physical division of the cells; this is in contrast with cell growth which involves an increase in cell size or mass. In order to identify a proliferating cell, these changes have to be detected and sensitivity problems similar to those associated with differentiation are encountered. The changes may be represented by discrete step-wise changes in the cellular concentration of, or by sharp peak alterations in, proliferation gene products. Many of these changes can be, and indeed have been, mapped on a time scale represented by the interval in time between two adjacent cell divisions (mitotic figures) i.e. mapped in relation to the *cell cycle*. A large number of the gene products of these proliferation-associated genes (which include many cellular oncogenes) have been mapped as transition points in the cell cycle. Traditionally the four major transition points: the onset and termination of DNA synthesis and mitosis have been used to identify proliferative cells but many other transition points may be equally valid. The search for sensitive and universely applicable markers for proliferative cells in other phases of the cell cycle continues (e.g. cyclin/PCNA studies, Bravo and Celis, 1980; Mathews *et al.* 1984; Galand and Degraef, 1989 and the nuclear antigens detected by Ki67, Miyachi *et al.* 1978; Gerdes *et al.* 1984, 1986, Verheijen *et al.* 1989).

There are certain difficulties in distinguishing cells on the basis of our definitions of differentiation and proliferation. The first thing to note about these two processes is that they are not necessarily mutually exclusive. Indeed many cells in the adult body may exhibit differentiation markers, and hence be differentiated relative to cells earlier in the tissue development and yet they also proliferate. Certainly many cells in the bone marrow exhibit both properties. Even the haemopoietic stem cells in the bone marrow are differentiated relative to embryonic stem cells. The stem cells in surface epithelia may be differentiated

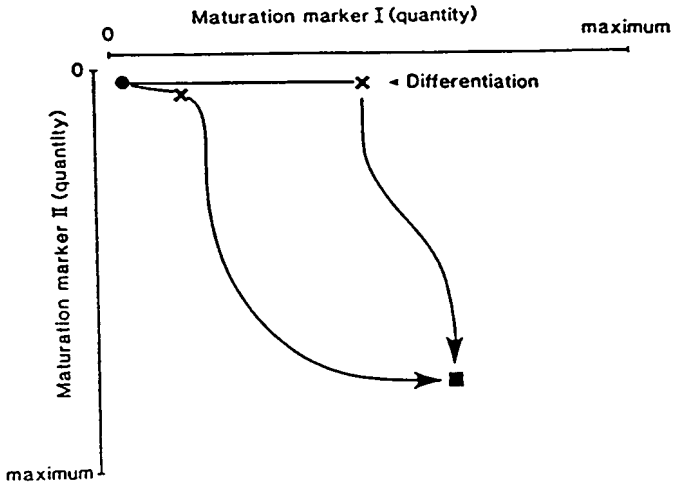


Fig. 1. The course of an individual cell can be described in a differentiation–maturation diagram. Aquisition of a qualitatively new marker is defined as differentiation (×), while the trajectory for a given marker (from ● to ×) or for a set of markers (from × to ■) is defined as maturation. Different maturation – differentiation paths may lead to the same state (■).

relative to the bone marrow stem cells and *vice versa*. The characterisation of the state of proliferation and differentiation are dependent upon the ability to identify changing patterns in gene expression and gene products. If these changes are of a cyclical nature they may be associated with proliferation. However, the cells under consideration may divide only once or we may have no knowledge of their previous history, in which case we are unable to tell if a particular gene product has been produced cyclically. Hence, it is more precise to define proliferation on the basis not of the cyclic appearance of gene products but the appearance of gene products associated with DNA replication, or the cell division process, which are in fact often produced in a cyclic fashion. This implies a knowledge of many or all the metabolic processes associated with, and leading to, cell division. Significant knowledge of these processes has been acquired (see e.g. Baserga, 1976, 1985, Pardee, 1987, 1989, Prescott, 1987; Kaczmarek, 1986).

The distinction between differentiation, maturation and proliferation appears important as the development from stem cells to functionally competent cells can be viewed as a transition from one extreme (prolif: yes; diff/mat: no) to the opposite extreme (prolif: no; diff/mat: yes). The transition takes place through states of coexistence with some flexibility to accelerate or slow down one or both processes. It is this flexibility that permits cells to be stimulated to differentiate and stop proliferation and *vice versa*.

(d) *Self-maintenance*

Selfmaintenance, selfrenewal, selfreproduction, selfreplication and selfregeneration are terms that have been used in connection with stem cells often interchangeably and without definition to the detriment of clarity. This had led to confusion and imprecision in understanding the concept of stem cells. These terms have subtle differences in meaning and should be used with care. *Maintenance* means 'to keep at an existing state or level' and when considered in terms of numbers is a meaningful expression to apply to a stem cell (see Lajtha 1979*a*, Potten and Lajtha, 1982). The ability to maintain its own numbers, i.e. selfmaintenance, is a property exclusively of stem cells. The term '*renewal*' can be defined as 'to make like new' which implies an element of rejuvenation. If selfmaintenance is satisfied, it implies that there is no input into the stem cell population from elsewhere and no renewal would be required. We would like to restrict the term renewal to a specific process to be discussed below. The term '*reproduction*' means to 'give rise to offspring' and is thus a property of proliferative cells. The term selfreproduction, however, implies that the offspring are identical in every sense, including genetically, with the parent, and is therefore a term best restricted to a budding or cell cloning processes. It is clear that selfreproduction has a stronger implication than selfmaintenance. *Replication* implies duplication or repetition and has connotations somewhat similar to reproduction. Selfreplication implies production of identical

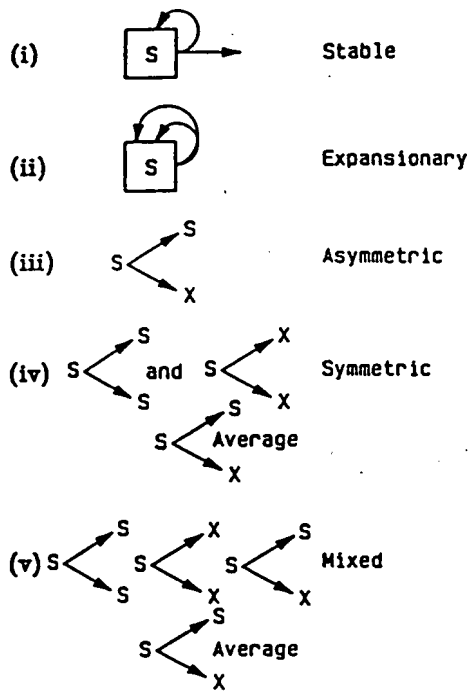


Fig. 2. Various considerations in connection with stem cell (S) divisions (see text). X=non-stem cell.

twins while selfmaintenance implies maintenance of a functional ability (e.g. number) irrespective of the identity. *Regeneration* implies 'to make again' something that was already pre-existing. It could apply to a tissue or a population of cells and would be more appropriately used in connection with other processes, to be discussed later, which exclude its use in connection with selfregeneration. So stem cells may be defined as cells capable of selfmaintenance and regeneration under certain conditions.

The principle of selfmaintenance and its implications are illustrated in Fig. 2. The terms selfreproduction or selfreplication could be applied to stem cells but in the interests of clarity we suggest using only the term selfmaintenance.

When a stem cell divides and both daughters remain as stem cells, the division could be regarded as *symmetric*; however, the stem cell population will have expanded i.e. the cells have been more than maintained (Fig. 2,ii). If by some mechanism one daughter at each division is removed the population is stable and the numbers are maintained. A number of possibilities exist to explain the division processes in stem cells. The situation where the output from the stem cell compartment to some other element of the tissue precisely equals the number of cells remaining in the stem cell compartment, we define as a *steady state*. This could be achieved in principle by a strictly controlled, regular, deterministic *asymmetric* division (Fig. 2,i and iii). In situations where stem cells are rare or very sparsely distributed and there is a need to minimise the risk of losing a stem cell through random death, a process of regular asymmetric division must apply if 'holes' or loss of tissue units is to be avoided. One of the questions

here is how the stem cells 'switch' from a regular asymmetric division process to one with some symmetric divisions if more stem cells are needed e.g. to compensate for the death of some stem cells. Detailed cytological analysis of cell divisions in corneal epithelium has identified both asymmetric and symmetric divisions in the basal layer but these cannot be related specifically to stem cells (Lamprecht, 1990).

One could also imagine a situation where the stem cells always divide to produce two similar daughters, i.e. the symmetric division process (Fig. 2,iv). In this case, steady state could only be achieved by some deterministic or stochastic process ensuring that on average half the stem cells produce two daughters that are not stem cells, thus producing an average situation that is equivalent to an asymmetric division. The type of division that is actually performed by an individual stem cell may be determined intrinsically (which leads to certain conceptual problems) or by the environment in which the cell finds itself. Changes in stem cell number could be achieved by simply altering the proportion of the two types of division. Consideration of this particular type of division scheme illustrates a particular problem associated with the stem cell concept. The problem arises from consideration of either individual cells or populations of cells, or how one defines the size of the compartment to be considered. If one looks at individual cells, a cell that produces two daughters that are not stem cells cannot itself be considered a stem cell since it does not satisfy the stem cell criteria of selfmaintenance. However, the definition is still applicable to a pool of many such cells contributing as a whole to the selfmaintenance process. This problem of compartment size relating to the stem cell definition reappears on numerous occasions in the consideration of stem cells.

A second problem here is a practical one in that the removal of cells to differentiation may occur very rapidly making it impossible to distinguish in practice symmetric division with rapid removal from asymmetric division. A final situation to consider in stem cell divisions is one where both types of individual symmetric, and the individual asymmetric divisions may occur (i.e. all three types of division in Fig. 2,v), and where under steady state conditions deterministic or stochastic processes determine that the average is equivalent to the asymmetric division process. Here factors that determine stemness and non-stemness would act on the population removing on average half the stem cells under steady state conditions. Such factors of course could be differentiation factors in which case the cells marked 'X' in Fig. 2 could be regarded as differentiated cells.

It is at this point that we need to introduce the concept of the *probability* of *selfmaintenance* p_{sm} which is a concept coming into play due to the practical and principal impossibility of determining the division scheme of an isolated individual stem cell. p_{sm} is the probability that cells within a sufficiently large population of cells will produce daughters like themselves. A population of cells with p_{sm} constantly greater than

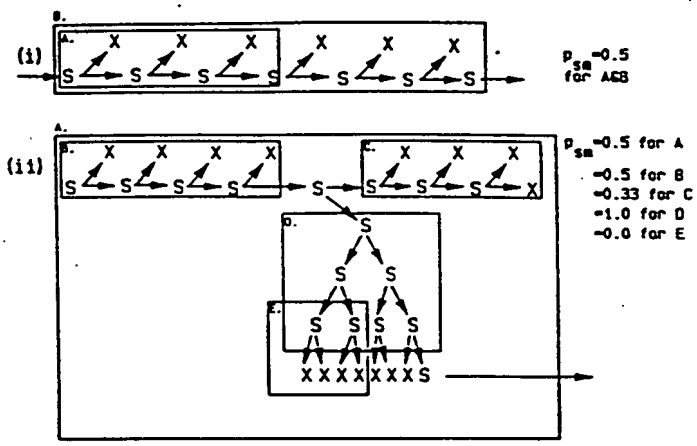


Fig. 3. Compartment size considerations. (i) Permanent asymmetric stem(s) lineage. Whatever compartment size is considered, selfmaintenance is satisfied. (ii) A second stem cell lineage. If all the divisions are considered selfmaintenance is satisfied (box A). Similarly for a selective smaller box (B) it is satisfied although a similarly sized different compartment (box C) does not satisfy these selfmaintenance criteria. Other compartments may show no selfmaintenance (box E) or maximum values (box D).

0.5 is expanding in size. A population of cells with p_{sm} constantly less than 0.5 is clearly declining in size with time and hence is not maintaining its numbers in the long run and therefore does not satisfy the stem cell criteria. According to the definition the characteristic of stem cells is that they can have a p_{sm} in steady state that equals 0.5 and that they have the ability to vary this from values below and above 0.5 under certain circumstances. Selfmaintenance of stem cells can be satisfied by any condition where p_{sm} is equal to 0.5 or greater if other processes remove cells. There is a specific experimental problem in determining the quantitative value for p_{sm} which is related to the pool or compartment size and observation time, respectively. If these are too small an estimation bias is very likely. This is illustrated in Fig. 3. Here, we have two cell lineages both characterised by predominantly asymmetric divisions that may be determined by any of the processes considered above. In the case of lineage 'i', whatever compartment size one considers even down to a single cell division, one could conclude that stem cells are involved. However, in lineage 'ii' the overall p_{sm} equals 0.5 (steady state), but there is a series of inappropriately small subcompartments for which p_{sm}, if evaluated, would be very different (box E: 0.0, box C: 0.33; box D:1.0).

(3) *Cell population compartments*

The simplest population compartment in connection with stem cells would be one which was purely expansionary in growth (see Fig. 2, ii). Such compartments may exist when an isolated stem cell is placed in culture, during early embryogenesis, and possibly under some conditions of wound or tissue repair (regeneration). It is possible to maintain the expansionary growth by removal of some stem cells which could

be achieved in principle by applying a simple spatial cut-off. As the cells reach a particular point in the tissue, for example the top of the intestinal crypt, they are instructed by some signal(s) to become mature functional cells. Such a *cut-off* could operate *via* some chemical signal from outside the crypt or by a chemical gradient of intercellular factors. In all tissues, functional cells are needed and in most cases a diversity of function is required, namely a specialisation of cells. In order effectively to achieve this diversity of function, the cells need to direct their energies and resources towards producing special materials or structures. On the whole this seems to preclude their continuing to undergo proliferation. Functional cells will require a certain time to manufacture their specialised proteins or structures. This introduces the idea of a maturation process following one or more differentiation events. Thus the transition from a stem cell to a mature functional cell is not an abrupt process. The introduction of a differentiation removal process from an exponentially expanding stem cell population implies an effective asymmetric division process for the population of cells (Fig. 2, iv,v). If the system is to attain steady state then the number of cells entering the differentiating compartment (M in Fig. 4,i) must equal the number of cell divisions in the stem (S) compartment. To our knowledge there are few (if any) mammalian tissues organised in this way, where there are only S cells and M cells. It is unclear why this is so. Perhaps the switch from proliferation to differentiation and maturation is not a simple change in genetic programs but requires time and even cell divisions (Holtzer, 1985). In a situation where there are only S cells and mature M cells, there is a large population of stem cells at risk from genetic error (see Cairns, 1975). One solution to these problems might have been to use the time that it takes for maturation in Fig. 4,ii for some continued cell proliferation e.g. Fig. 4,iii. Such a dividing, maturing, cell population allows much of the work load in terms of cell production to be removed from the stem cell compartment which as a consequence becomes much smaller and hence offers a smaller target for genetic and carcinogenic damage. This maturation time also allows for the generation of diversity of function (i.e. additional differentiation events) whether this requires rounds of cell division (quantal cell cycles, Holtzer, 1978, 1979) or some other mechanism remains uncertain (Lajtha, 1979*b*).

This model introduces a new class of proliferative cells the *dividing transit population (T)* (see Lajtha 1979*a*,*b* and Gilbert and Lajtha, 1965). The concept implies that, wherever a high cell production rate is required, a T population might be expected and the higher the cell production rate the more cell divisions could be expected, in the T population (see Cairns, 1975; Lajtha 1979*b*, Potten and Lajtha, 1982). The converse might also be expected, if turnover is extremely slow the tissue could in principle operate effectively with just stem cells and maturing differentiated cells. Convincing examples of this type of organisation are lacking.

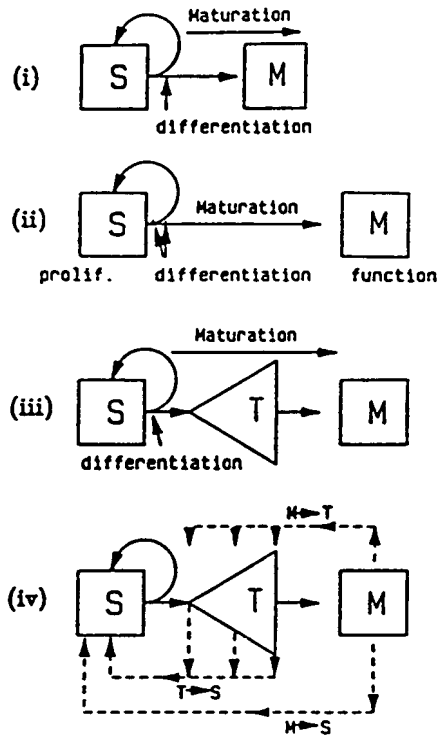


Fig. 4. Consideration of the process of maturation of non-stem cells (M=mature functional cell). It is unlikely that the process of differentiation leads instantly to the production of a mature functional cell (i) i.e. maturation requires a certain time (ii) Differentiation may occur at division or at some time after division. During maturation, but after differentiation, some cell proliferation may be possible in a dividing transit (T) population (iii). The existence of a T population increases the range of possible feed-back controls (iv); the larger the T compartment the greater the need for intermediary feedback loops to minimise excessive fluctuations in cell output.

It is clear from the scheme represented in Fig. 4,iii that the cell production rate, i.e. the number of M cells produced per unit time is determined by the number of stem cells considered, their cell cycle time and the number of cell divisions (cell generations) in the T compartment. The advantage of the transit population is that it enables some genetic protection to be afforded to the stem cells. It effectively amplifies each stem cell division thus minimising the number of stem cell divisions required, and hence conserves the stem cell energy and genetic load. It also allows for diversity of specialisation at a low cost in terms of proliferation and genetic risk for the stem cells. The disadvantage of such a hierarchy is that the length of time spent in the T population can result in an instability in cell output following damage (i.e. overshoots and fluctuations). This can be overcome by introducing feedback loops and dampening phenomena, such as a high variability of cell cycle or transit times (Wichmann *et al.* 1988). The total cell output can be controlled by either the number of cell generations in the T compartment, which might be controlled for example by a feedback loop from the M compartment, or by controlling the output from the

stem cells, i.e. their cycle time. If there are many T generations, there is a logistic problem in terms of the spatial distance in a tissue over which a feedback loop would have to operate from the M population to the stem population. This may be overcome by growth factors that operate over long distances (i.e. hormones) or a breakup of the system into several feedback loops. Such feedback loops are illustrated in Fig. 4,iv.

(a) *The dividing transit population*

It is implicit from Fig. 4,iii,iv that the dividing transit population can be distinguished from the stem cells by a differentiation event and a spectrum of increasing maturation. The transit cells are a transition population of proliferating cells situated between the stem cells and the mature functional compartment. Thus they would be expected to share some properties with stem cells and some properties with mature cells. The stem-like properties might be expected to decline as the M-like properties increase. The main property that they share with stem cells is the ability to reproduce or replicate. The property they share with mature cells would be the acquisition of one or more differentiation markers. A primary feature of transit cells is that they are inexorably destined to move towards functional status. This can be represented, as in Fig. 5, by a cell moving down a spiral pathway of increasing maturation/differentiation, a concept with similarities to the continuous maturation/proliferation model of Mackey and Dörmer (1982).

The situation represented in Fig. 5 is one of two alternative possibilities for the T population. It shows a discrete (quantal) change from the stem to the transit population and although the T population in Fig. 5 retains the property of division in common with the stem compartment, it cannot selfmaintain (stay at the same level of maturation/differentiation). The T population is entirely dependent on an input from the stem compartment. If the stem compartment is removed or destroyed the T population will disappear and has no possibility of maintaining the tissue or itself. In this particular model, only the stem cells have the ability to regenerate the T population and the tissue.

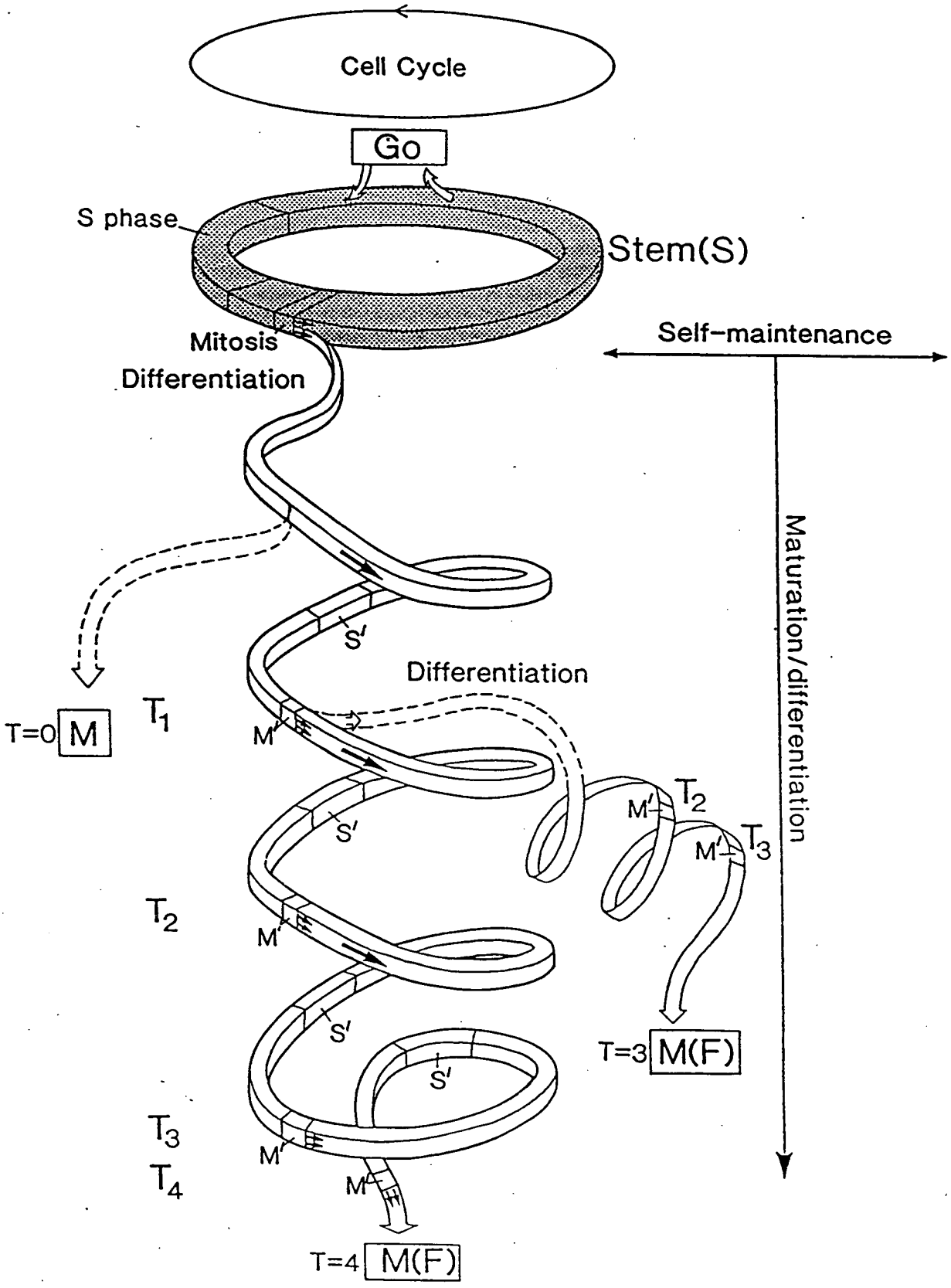
The second and more realistic possibility for the T population is illustrated in Fig. 6, which suggests that the T compartment retains some additional attributes of stemness i.e. they possess a progressively declining spectrum of stemness and an increasing spectrum of differentiation and maturation. This model suggests that the earlier transit compartments retain a certain ability for selfmaintenance, i.e. some cells do not progress down the spiral (that is mature) but remain for at least one cycle at the same level. This property may be retained to a lesser extent in the second and third generation (at declining levels). In this case, the feature that distinguishes a transit cell from a stem cell is not so much the question of whether or not it can selfmaintain but its maximum capability of selfmaintenance. A transit cell population will by definition always have a p_{sm} value less than 0.5 under steady state conditions. Its ability to vary its p_{sm} may be considerable but restricted and certainly declines with increasing maturity. The advantage of this model is that some T cells are very similar to the stem cells or indeed indistinguishable from stem cells in some situations but, although these cells may have an ability to behave like stem cells (i.e. $p_{sm} \geq 0.5$) in some special circumstances under normal steady state conditions, they do not. The necessity for considering such a model comes from regeneration experiments *in vivo* particularly those in the small intestine (see Potten *et al.* 1987). It should be noted that the spiral model is a much more comprehensive model of the A–T–M scheme than the compartment concepts in Fig. 4 because it inherently allows for description of a wide variety of different individual cell developments (trajectories). In this respect, it is an extension of the concept of an hierarchical tissue organisation (Michalowski, 1981; Gilbert and Lajtha, 1965; Potten, 1974; Loeffler *et al.* 1987; Clausen and Potten, 1990; Potten 1983*a*,*b*, Potten and Hendry, 1983; Potten and Morris, 1988; Potten *et al.* 1979, 1982*a*,*b*, 1983, 1987). The cell cycles illustrated in Fig. 5 allow the compartment size phenomenon that we have already discussed to be illustrated. If a single cycle in a T compartment is considered, the selfmaintenance probability is not meaningful. However, by considering the entire T compartment an element of selfmaintenance may be applicable but this will be less than 0.5. In Fig. 6 there is a true element of selfmaintenance (always less than 0.5) for several of the T generations. The selfmaintenance probability can be represented in Fig. 6 by a series of arrows indicating, by the number of arrows and the length of the arrows, the range of possibilities for cell trajectories and the most likely direction that a cohort of cells will take in relation to selfmaintenance on the horizontal scale and differentiation and maturation on the vertical scale.

This scheme has certain similarities to the niche and declinining G_0/stem cell quality theory proposed by Potten *et al.* (1979 – see Fig. 7 and Potten and Lajtha 1982). The relationship between the stem cells and the transit generations and their position in the crypt (distance from some focus, niche or extracryptal environment) could determine their functional abilities.

The terms committed stem cells or early progenitor cells are sometimes used to denote proliferative cells that possess some limited degree of selfmaintenance. Such quasi stem cells clearly are the same as the early transit cells in the diminishing stemness spiral in Fig. 6. Whether or not such cells are justifiably called stem cells depends on the time frame being considered. Over the entire life of the animal they do not act as stem cells but over a period of several cell cycles they may satisfy the stem cell definition providing they selfmaintain. It is only if they undergo renewal that they can truely be said to be stem cells.

(D) Problems in measuring stem cells

If we consider the stem cell definition presented earlier, the question arises as to whether it can be used in a



practical sense. A stem cell is a proliferative cell but this is the weakest part of the definition. Proliferation can be identified in a population strictly only by determining the future behaviour of the cell in question, i.e. will this particular cell divide into 2 cells in the future, if it does it is a proliferative cell. In practice it is usually sufficient to identify that the cell, or population of cells, express one or more of the many markers of transition

Fig. 5. Diagrammatic representation of the stem cell(s) and dividing transit (T_0–T_4) cell populations (simplified scheme):– the *abrupt differentiation–maturation spiral.* Proliferation is represented by the horizontal cylindrical axis and differentiation–maturation by the vertical linear axis. Selfmaintaining stem cells (S) remain at the same horizontal level. They produce by division other selfmaintaining cells and by differentiation dividing transit (T) cells. Such cells are then inexorably destined to fall down the maturation spiral, dividing as they fall to eventually produce mature functional cells (M,F). Additional differentiation events could occur giving rise to separate spirals. The number of divisions (T_0–T_4) may vary from zero upwards. Some stem cells may enter, and leave, a quiescent (G_0), non-cycling state. The diagram could be regarded as indicating the path of an average T cell during maturation. The spiral or corkscrew provides the guiding path for an average T cell like a fun-fair helter-skelter. The predominant feature of this model is the sharp, irreversible, distinction between stem cells and transit cells. The only feature these two classes of cells have in common is their ability to divide. Although the stem cells divide according to some process asymmetrically (see Fig. 2), the transit cells divide symmetrically and each division (generation) can be distinguished by its maturity. The S phase (S') and the M phase (M') of the cell cycle are represented on the cycle and spiral. The height and thickness of the ribbon describe the paths of a small cohort of cells. For realistic cohorts, one can expect an increasing broadening of the spiral as the variance of the cell cycle and maturation velocities becomes more important. This model description is similar to the one given in Fig. 4(iii).

through the cell cycle. The commonest one of which is whether it is in the DNA synthesis phase but the simplest of which is whether it is in the mitotic phase.

The second aspect of the definition is whether or not the cell is undifferentiated. This is a qualitative and relative term. It would usually be assessed by observing the morphological status of the cell and whether or not it expresses one or more markers for differentiation.

The next aspect of the stem cell definition relates to the ability of these cells to produce a large progeny of differentiated cells. This again is a question related to the future potential of the cell or cells in question and can only be tested by placing the cell or cells in a situation where they can express this potential, for example, placing the cells in culture or arranging for a situation *in vivo* where the regulators limiting stem cell growth are removed as would happen in a situation where stem cells were killed i.e. during a regeneration. We will return to the question of regeneration.

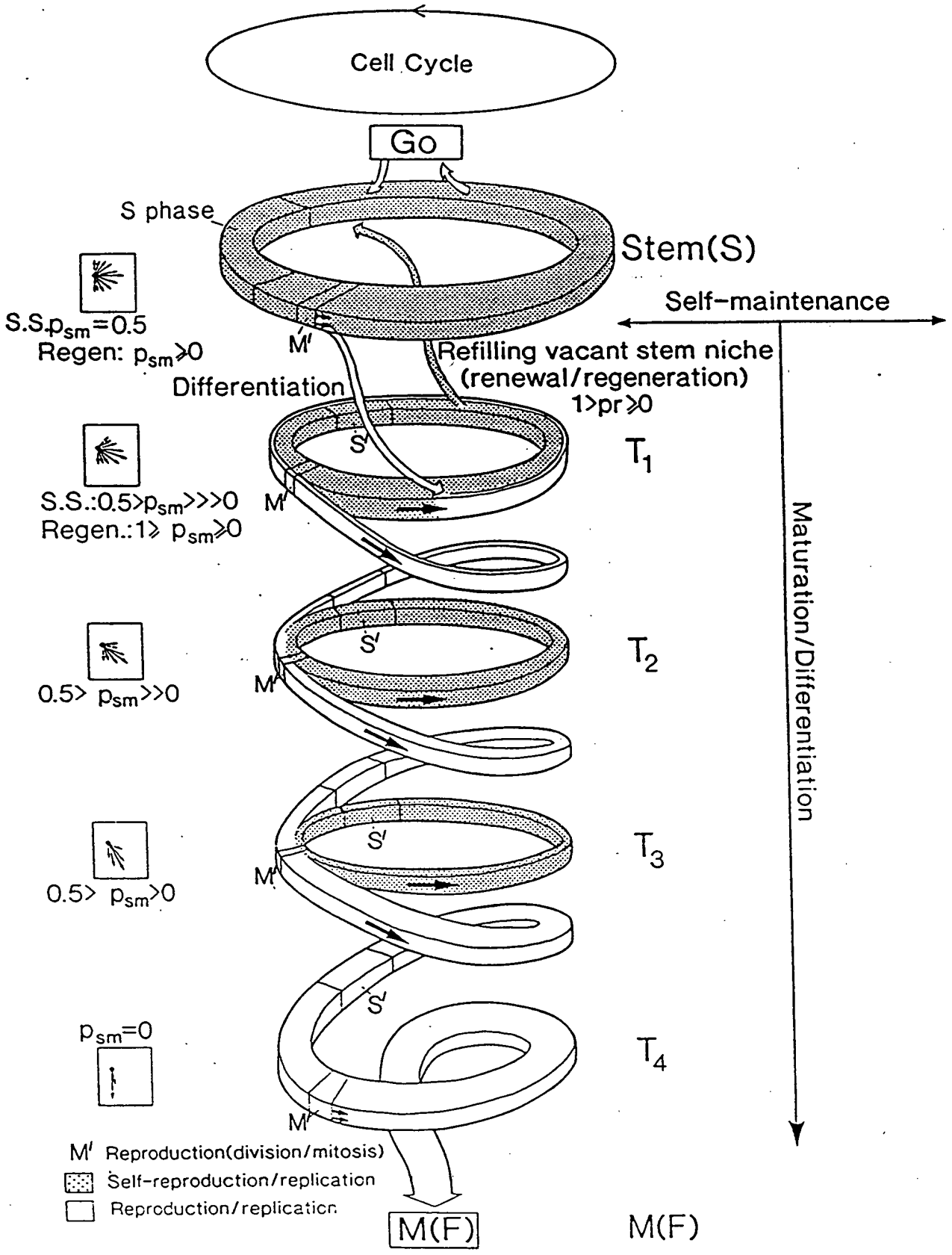
Selfmaintenance is another cardinal property of stem cells, but here again it can only be assessed in terms of the future of the cell or population. In other words, can the cell produce other cells like itself and can the population maintain itself over a period of time. Changes in selfmaintenance probability can similarly only be measured with the passage of time.

The final aspect of the stem cell definition, which is associated with the property of a large proliferative potential is whether regeneration can be achieved. Besides being a property associated with the future this is specifically a property associated with a disturbance of the system.

As can be seen from these considerations, the main attributes of stem cells relate to their potential in the future. These can only effectively be studied by placing the cell, or cells, in a situation where they have the opportunity to express their potential. Here, we find ourselves in a circular situation: in order to answer the question whether a cell is a stem cell we have to alter its circumstances and in doing so inevitably lose the original cell and in addition we may only see a limited spectrum of responses. This situation has a marked analogy with *Heisenberg's uncertainty prinicple* in quantum physics. In simple terms, this states that the very act of measuring the properties of a certain body inevitably alter the characteristics of that body, hence giving rise to a degree of uncertainty in the evaluation of its properties. The analogy holds true for the functional stem cell assay procedures, all of which observe the response after a perturbation to the system thereby challenging the different capabilities of the cells in different though complementary ways. Therefore it might be an impossible task to determine the status of a single stem cell without changing it. Instead one would have to be satisfied with making probability statements based on measurements of populations. The uncertainty principle in quantum physics makes a similar statement, that the status of a quantum particle cannot be determined with accuracy leaving only probability statements meaningful.

In practice, clonal growth assays are a frequently used way of assessing stem cell function. For adult tissue stem cells, a variety of clonal regeneration assays have been developed *in vivo* and a number *in vitro* (summarised in Potten and Hendry, 1985*a*,*b*). The most effective of these is the spleen colony assay for haemopoietic stem cells (Till and McCulloch, 1961) and the micro and macro colony assays for intestinal epithelium (Withers and Elkind. 1969, 1970, Potten and Hendry, 1985) and epidermis (Withers, 1967). In these cases, the colonies assessed are large and contain very many cells and, if the necessary conditions are satisfied (high doses of radiation), represent *clones* derived from a single stem cell. The fact that these clones contain many cells demonstrates the large division potential of the originator cell (*clonogenic cell*). The fact that the clones can often contain several differentiated cell lineages indicates that the original stem cell possessed the ability to provide cells that differentiate in a variety of ways. The selfmaintenance element can be assessed by either a second clonal regeneration assay starting with the first clone, or simply from the longevity of the clone in terms of maintenance of its cellularity and differentiation and the fact that it eventually repopulates an entire area of the tissue.

If we consider the scheme in Fig. 6, the stem cells within this figure are clearly capable of clone formation and regeneration. A major question is whether any of the T population under conditions of severe cellular depletion could regenerate the epithelium. If this could occur it involves a sort of rejuvenation process of the T



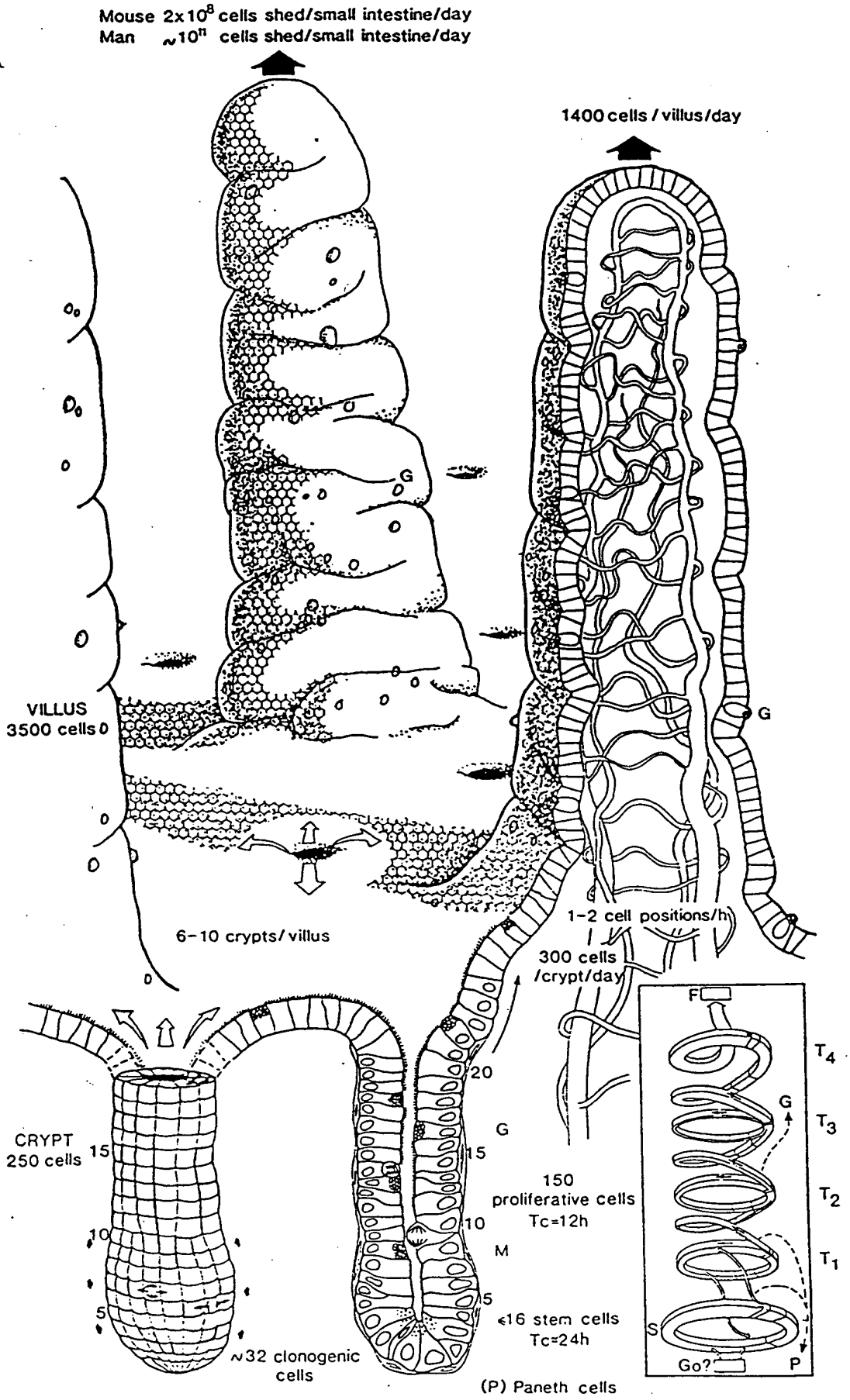
cell, or a renewal of a T cell, i.e. its return to the status of a fully effective stem cell. Thus both stem cells and T_1 cells may be capable of *regeneration* of the tissue but it is only T_1 cells that undergo a process of *renewal*. Clearly if T_1 cells really represent a spectrum of declining stem cell properties then these questions could in principle be asked concerning the T_2 population and so on. If we regard the T_1 population as

Fig. 6. Diagrammatic representation of the stem cell (S) and dividing transit cell populations (T) (general scheme):- *the diminishing stemness spiral.* Proliferation is represented by the horizontal cylindrical axis and differentiation–maturation by the vertical axis. See Fig. 5, for further details. As is the case in Fig. 5, additional differentiation pathways could occur at any level in the spiral. The distinction between this model and that shown in Fig. 5 is that the transit cells represent a truly intermediate cell population between stem and mature (M) or functional (F) cells possessing characteristics of both. Hence, the transit cells (T) retain some ability for selfmaintenance (horizontal cell cycles as opposed to spiraling cycles). The probability of selfmaintenance is always less than that for the stem cells ($p_{sm}<0.5$) and declines with each transit generation. Thus the probability of a cell progressing down the spiral increases from >0.5 at T_1 generation to 1.0 at the T_4 generation. In a real system, the height and width of the ribbons will be much broader and frequently overlapping due to variation in cell cycle times, maturation velocities and selfmaintenance processes. Since the T_1 population at least possesses some selfmaintenance ability, it can be regarded, when under selfmaintenance cell cycle conditions, to be indistinguishable from stem cells and if a vacant space becomes available in the stem cell environment (niche – see Schofield, 1978) then such a T_1 cell could reoccupy a vacant niche and become an actual stem cell. This represents a sort of rejuvenation or renewal. Hence renewal is a process that is unique to some transit cells and the probability of renewal is p_r. Having renewed, such a cell could then regenerate the stem compartment, the differentiation spiral(s) and the tissue. Regeneration is thus a property unique to stem cells. The T_1 cells here could be equivalent to the committed stem cells described in some cases. They are also potential stem cells as they have the possibility of renewal. At each level in this diagram, the selfmaintenance probability (p_{sm}) changes as does the range of options open to a cell. These are diagrammatically represented by the boxed arrows on the left. It is important to realise that since this diagram represents the possible path of a cell in time where there is a bifurcation in the path (cell division in the T_1–T_3 population) the two paths represent the extremes of the options open to individual cells. S.S.=steady state; regen.=regeneration; S'=S phase; M'=M phase. The spiral shown here has two dimensions, one representing selfmaintenance (horizontal) and one representing maturation and differentiation (vertical). These two axes do not relate to the three-dimensional spatial axes of the crypt.

capable of re-entering the stem cell cycle then they constitute a class of *potential stem cells* as distinct from those that are performing stem cell function which we would term *'actual' stem cells*. (A second class of potential stem cells would be those that had actually stopped progression through the cell cycle and therefore were in some *quiescent or G_0* phase that could at any moment be recalled into proliferation.) We would define the *renewal probability* of a transit cell population as the likelihood that it rejuvenates itself such that it could perpetually selfmaintain and retain the capability of tissue regeneration.

However, the difference between stem cells(s) and T_1 transit cells may be very small and it might be difficult to prove the renewal process on a molecular basis because it would involve the demonstration that an activated T_1 differentiation marker disappears under certain circumstances. On the other hand, there is good experimental evidence in hemopoietic and epithelial systems that fully competent tissue regeneration is still possible even after frequent severe damage (e.g. chronic or fractionated irradiation or drug application). The scheme in Fig. 6 therefore summarises, in a fairly general sense, the different views on the generation of hierarchical cellular systems and the ways of allowing flexibility. Control mechanisms may act on the proliferation velocity, maturation velocity, differentiation and selfmaintenance probabilities (and possibly on renewal processes) of stem and transit cells. Thus, for example, the known haemopoietic growth factors G-CSF and EPO can be interpreted to act on cell cycle progression and on p_{sm} of fairly mature transit cells (e.g. T_2 in Fig. 6). Thus they can induce a greater cell amplification but ultimately the cells will progress to full maturity. The control processes involved in the stem–T_1 region are still poorly understood but it should be important to distinguish the different target parameters of control: proliferation, (cell cycle, G_0) from differentiation–maturation (selfmaintenance, renewal). Fig. 6 also indicates some of the difficulties in determining stemness because, depending on the criteria applied (undifferentiated, or selfmaintenance, or regeneration ability), different subsets of the spiral are identified.

Stem cell identification would be greatly facilitated by the development of appropriate markers specific to stem cells or specific to transit cells. However, in practice such an identification of stem cells results in the same problems of uncertainty and compartment size as discussed previously. If the compartment size is equivalent to the entire tissue one can decide relatively easily whether or not the compartment contains stem cells. Consequently one reduces the compartment size and using the same markers asks the question again. At each step one can only say that the compartment either contains stem cells or it does not and the only firm conclusion is the situation where you can say that it does not. Eventually the compartment size might be the same as an individual cell, in which case the uncertainty principle enters and you can only assess the cell by changing its situation. There is no situation where one can say with absolute confidence that the entire compartment is composed of only stem cells unless one does some test for the future potential of each of the individual cells. An important cautionary consideration here concerns the rigour of the test placed on cells. It is possible that tests could be applied that are so far from the normal experiences encountered by cells *in vivo* that misleading results could be obtained. All cells, including mature functional cells, contain the same DNA and the cell could be experimentally manipulated to unmask, derepress and activate any part of its genome including that for cell division or even that for stemness but such manipulations provide no infor-


Mouse 2x10^8 cells shed/small intestine/day
Man ~10^11 cells shed/small intestine/day
1400 cells / villus/day
VILLUS
3500 cells
6-10 crypts/ villus
1-2 cell positions/h
300 cells
/crypt/day
CRYPT
250 cells
150
proliferative cells
Tc=12h
≤16 stem cells
Tc=24h
~32 clonogenic
cells
(P) Paneth cells
F
G
S
Go?
P
T4
T3
T2
T1
20
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M

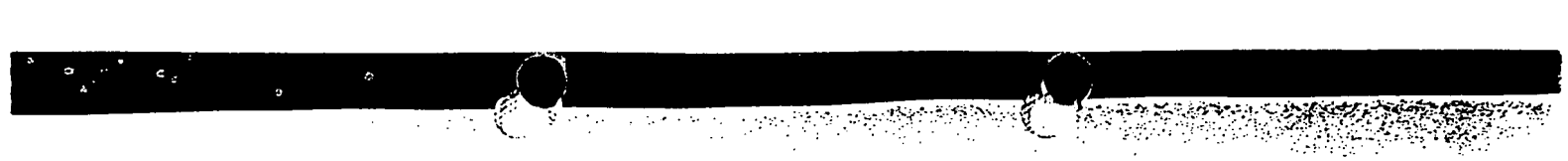

Fig. 7. Diagrammatic representation of the three-dimensional gross architecture, histological cell organisation and cell kinetic hierarchy for the ileum of the mouse. The diagram has been modified and adapted from Potten and Loeffler (1987), and Potten and Morris (1988). In longitudual sections, the cells in a crypt can be identified by their position (1–20). P=Paneth cells. T_c=cell cycle duration. G=goblet cell. G_0=quiescent cells. The capillary network in the villus is shown on the right. The stem/transit (T_1–T_4) spiral is shown, bottom right (see Fig. 6).

mation on whether in real life the cells actually ever express these genes.

Previous functional assays of stem cells were often clonogenic assays. A *clonogenic cell* is a cell that is capable of producing from one cell a large number of progeny, i.e. a clone (see Potten and Hendry, 1985). This is really only a technical aspect of stem cell measurement in some specialised circumstances. If the clone can be demonstrated (usually by secondary clonogenic assays) to contain further clonogenic cells then selfmaintenance has been satisfied. Clonogenic cells thus may satisfy some of the criteria for stemness e.g. clone formation and selfmaintenance. However, it is often not clear whether clonogenic cells are able to regenerate the entire tissue because they are assayed fairly soon after the damage. This raises the question of whether clonogenic cells are a mixture of stem and early transit cells. It is most likely that clonogenic assays measure all, or a part, of the potential stem cells which may be a considerable overestimation of the number of actual stem cells.

(E) Pluripotency of stem cells

In the stem cell definition given above, pluripotency was not presented as a prerequisite of stemness. However, it is clear that most tissues contain a range of different specialised functional cells. These may all originate from a common compartment of stem cells in the tissue, the range of variable different differentiation options being facilitated by the length of the transit compartment. Those tissues with the greatest differentiation potential, for example bone marrow, tend to have the longest transit compartment. The limit to the differentiation potential for individual stem cells is unclear and may well differ from tissue to tissue. The ability to produce progeny that differentiate down various lineages (pluripotency) is not necessarily a property of stem cells *per se*, although it appears that many stem cells possess this capability. It should be recognised that many more options for differentiation than are normally expressed might be possible. The limits are unknown e.g. it is not known whether under special conditions bone marrow stem cells might be capable of making even more different cell lineages than are normally attributed to them – could they even make skin or gut? (see cautionary note above regarding the rigour of experimental tests). Neither is it completely clear whether cell divisions are always needed for sequential differentiation events (quantal cell cycles, Holtzer, 1978).

In the light of the spiral model (e.g. Fig. 6), we consider it likely that increasing maturation in the transit cells progressively restricts the possibility of allowing new differentiation events to occur. This may be linked to the continuous loss of flexibility e.g. of selfmaintenance and proliferation. It is an interesting speculation to argue that pluripotency may be linked to the uncertainty phenomenan.

(F) The crypts of the small intestine

The crypts of the small intestine are small, flask-shaped epithelial structures containing about 250 cells belonging to four cell lineages (columnar entrocytes, mucus-secreting goblet cells, Paneth cells at the crypt base, and the infrequent enteroendocrine cells). About two thirds of the cells (150–160 per crypt) can be seen to be passing rapidly through the cell cycle i.e. are proliferative. In fact they divide about twice a day so the crypt produces about 300 new cells each day. Cells leave the top of the crypt with a velocity of about 0.75 cell positions or cell diameters per hour and since there are about 16 cells in the circumference of the crypt this gives a figure of 12 cells produced (and hence leaving) the crypt per hour. The cell kinetics and cell organisation of this system have been extensively reviewed elsewhere (Potten *et al.* 1983; Wright and Alison, 1984; Potten and Morris, 1987; Potten and Hendry, 1983) and are summarised in Fig. 7.

The velocity of the cell movement decreases linearly with decreasing position in the crypt (Kaur and Potten, 1986). In transverse sections of the intestine, many crypts will be longitudinally sectioned, in which case they will contain about 25 cells on each side (each crypt column). However, detailed studies illustrate that the crypt actually contains about 16 cells in circumference and about 16 in column height (Potten *et al.* 1988*a*). The extra 9 cells seen in the column height result from the section width and the packing arrangements of the individual cells (Potten *et al.* 1988*a*). Longitudinal sections of the crypts permit a detailed cell position by position analysis of cell proliferation/differentiation behaviour. A variety of studies have used this approach in the past (Cairnie *et al.* 1965; Al-Dewachi *et al.* 1975, 1977, 1980) and we have used it extensively over the last few years to study a variety of proliferation or differentiation related end-points (Kaur and Potten, 1986; Ijiri and Potten, 1983, 1987, Potten *et al.* 1982*a*; 1988*a*, 1990).

(a) *Number of stem cells per crypt*

It is clear that the highly specialised functional Paneth cells do not satisfy any of the criteria for stem cells. Neither do the cells in the upper region of the crypt or on the villus, which apparently do not express any proliferation markers. This however, leaves about 150–160 proliferative cells. This compartment clearly contains stem cells since the crypt is selfmaintaining

over a long period of time (several hundred days in the mouse). If we consider a narrow compartment near the top of the crypt, it is clear that the criteria of selfmaintenance and a large division potential are not satisfied. Such geometric or positional considerations lead one to conclude that the crypt contains a ring of about 16 cells near the bottom of the crypt but above the Paneth cells (at about the 4th position from the bottom) that contains the *actual crypt stem cells*. Such considerations have been discussed by numerous people in the past (Leblond and Cheng, 1976; Cheng and Leblond, 1974; Potten *et al.* 1983; Potten and Hendry, 1983; Wright and Alison, 1984; Potten and Morris, 1987).

An ingenious clonogenic assay was devised by Withers and Elkind in 1969 which has since been used fairly extensively by many workers (summarised in Potten *et al.* 1983; Potten and Hendry, 1983; Potten and Hendry 1985*a*,*b*). The results of these experiments have been interpreted to provide an estimate for the number of cells with a clonogenic capacity i.e. the number of *potential stem cells per crypt*. These numbers tend inherently to possess large error limits and to be somewhat variable. The current conclusion is that the crypt contains 30–40 of these clonogenic cells (Potten *et al.* 1987, 1988*b*). Hence about 120–130 of the crypt cells are proliferative but not clonogenic or potential stem cells and are therefore cells in the dividing transit population. A recent study using prometheium β irradiation of exteriorised segments of intestine (Hendry *et al.* 1989) has shown that the weak β particle irradiation of the bottom of the crypt sterilises the structure and hence the largely unirradiated upper crypt cells do not possess the potential to regenerate the epithelium, which independently confirms the conclusion above. These studies in fact suggested that the number of potential stem cells may be less than 16; the actual best fit to the data being about 3 per crypt at about the 5th cell position from the base of the crypt. The conclusion that can be drawn at the moment is that an annulus of cells at around the 4th position from the base contains up to 16 actual stem cells. The annulus at the next highest position, the 5th would then presumably contain the T_1 population, some or all of which may represent potential stem cells (Potten *et al.* 1987). Thus, for this system, there is little evidence in support of any degree of selfmaintenance for T_2 or T_3 cells (see Fig. 6).

Scattered amongst the Paneth cells are about 10–14 undifferentiated intercalated (or crypt base columnar) cells. It has been suggested that these may be either actual crypt stem cells (Bjerknes and Cheng, 1981), or part of the actual crypt stem cells. Alternatively these cells may be part of the Paneth cell lineage. Indeed it is even possible that they represent an entirely separate population of stem cells: the actual stem cells for the Paneth cells. However, these intercalated cells do not apparently possess gap junctional communication with the Paneth cells (Bjerknes *et al.* 1985). We feel that it is unlikely that they represent pluripotent actual crypt stem cells because they rarely appear as vertical pairs (Chwalinski and Potten, 1989) and they do not apparently displace functional Paneth cells to higher positions in the crypt. Thus, unless they possess some remarkably versatile movement abilities, they cannot be the origin of the predominant crypt columnar cells.

It is interesting in connection with Figs 6 and 7 that gap junctional communication appears to be most effective (most rapid transfer of the Lucifer Yellow dye) in the stem cell zone. It declines with increasing cell positions from the crypt base (Bjerknes *et al.* 1985). It is also at the crypt base where mathematical modelling (Loeffler *et al.* 1986, 1988, Potten and Loeffler, 1987) has suggested that the greatest range of different cell generations (greatest age disorder) is to be found. In order to achieve the ordered distribution of cell ages seen at the higher cell positions some ordering process (communication) would be required in the lower crypt.

The indications from a variety of different experiments are that the actual stem cells of the crypt are capable of producing all four differentiated cell lineages within the crypt, i.e. they possess an element of pluripotentiality for differentiation. The evidence comes from detailed histological studies of radioactively marked cells and a knowledge of the turnover time of the 4 cell lineages (Cheng and Leblond, 1974): and from irradiation studies where all 4 differentiated lineages are observed in clones derived from a single surviving potential stem cell (Potten unpublished observations, Inoue *et al.* 1988). In the latter case, the clonality was confirmed by using an X-linked enzyme (phosphoglycerate kinase) and heterozygous F_1 mice.

The lower limit for the possible number of actual stem cells per crypt is clearly one. In which case the single cell in the small intestine presumably would be located somewhere in the fourth cell position annulus. There is an interesting strategic problem associated with the positioning of a single stem cell in the small intestinal crypts. If it is placed in the annulus at cell position 4, it is hard to see how an asymmetry in crypt proliferation is avoided. If the single cell is placed at lower positions, the migration of columnar daughter cells must be very complex in order to avoid the displacement of Paneth cells.

Studies with mouse embryo aggregation chimeras and lectin binding to visualise a mosaic staining pattern show that during development each crypt appears to be derived from a single stem cell since crypts heterogeneous for the lectin staining are not seen beyond the fourteenth day after birth (Ponder *et al.* 1985; Schmidt *et al.* 1988). However, with further development, the crypt may continue to remain dependent on one actual stem cell, or the original stem cell may expand to give up to 16 actual stem cells. Studies by Winton and coworkers (1988, 1989) using the mutagen ethylnitrosourea (ENU) using a mutation-induced cellular lectin marker in F_1 hybrid mice show that some crypts express both mutated segments and normal segments. These mixed crypts take a long time to become homogeneous or monophenotypic i.e. adopt uniformly either the wild-type (unrecognisable) or mutated phenotype (Winton, personal communication). These studies can be interpreted as suggesting that the crypts single actual

stem cells that can suffer mutation and which, given time, will eventually colonise the entire crypt with a clone of cells including new stem cells expressing the mutated phenotype. Similar studies have been performed in the colon using X-linked enzyme markers in female F_1 mice where uniformly mutated crypts are again observed (Griffiths *et al.* 1988). However, such studies are not without their interpretative difficulties. There may be more than one actual stem cell per crypt with several being very sensitive to the cytotoxic effects of the mutagens used. The single mutated clone may be derived from a single surviving resistant mutated stem cell. However, observations in untreated animals show that monophenotypic crypts accummulate throughout life. Furthermore, if the process of 'cleaning' a mixed mutated crypt to a homogeneous phenotype takes a time much longer than the cell turnover time in the crypt, and this is the actual case, then other possibilities have to be considered, e.g. that several actual stem cells must exist per crypt or that there is a single stem with an unusually long cell cycle. Mathematical modelling studies have shown that a mutation in 1 out of, for example, 4 or 8 stem cells can overgrow the other 3 or 7 and take over the crypt by purely chance phenomenon if one assumes stochastic processes involved in stem cell division with symmetric and asymmetric stem cell divisions (see Fig. 1,v) (Loeffler and Grossmann, 1990). On the basis of this model, it is possible to predict the time scale of the cleaning process of mixed mutated crypts to homogeneity. For crypts with e.g. 6 actual stem cells and 10% symmetric and 90% asymmetric divisions, the model predicts a time in the order of 100 days. This is compatible with data recently obtained (Winton and Ponder, 1990). Another alternative interpretation would be that the crypts contained a single actual stem cell with an exceptionally long cell cycle which then gives rise to daughter cells that rapidly repopulate the crypt. These daughter cells then maintain the crypt during the stem cell intermitotic time. There is at present no actual experimental evidence in support of such cells, but they would be very difficult to detect. Modelling exercises have shown that a single slowly cycling stem cell would result in large fluctuations in time in the size of the proliferative compartment of the crypt and in long tails to the right in the labelling index *versus* cell position plots. These are not observed.

The process of crypt fission has recently been linked to the number of actual stem cells present in a crypt (Totafurno *et al.* 1987). It was shown by mathematical modelling that one can obtain a quantitative fit to crypt size distributions, crypt fission and extinction rates if one assumes that exceeding a certain threshold number of actual stem cells leads to crypt fission. If one assumes the stochastic stem cell division process just mentioned, the observations are compatible with 4 to 16 actual stem cells (Loeffler and Grossmann, 1990).

The question of the precise number of stem cells, their cell cycle time and their location in the crypts remains somewhat uncertain. The number of stem cells and their cycle time are, to at least some extent, related and these two parameters determine the number of transit generations when an observed number of cells are produced per crypt (see Loeffler *et al.* 1986; Potten and Loeffler, 1987). When considering these points and the modelling of crypt cell replacement we are faced with the problems of satisfying Occam's razor. This much simplified states that the smaller the number of assumptions made in explaining adequately a phenomenon the better. The problem with the crypt is what is the simplest concept (least assumptions) that explains the crypt organisation and cell replacement which is consistent with the data. We believe that the situation can best be summarised as follows; the crypt contains between 4 and 16 actual stem cells with cell cycles between 12 and 32 h. There are up to 30–40 potential stem cells and between 4 and 6 transit cell generations. The stem cells are most likely to be arranged in an annulus at the 4th cell position from the crypt base.

The model shown in Fig. 6 helps in reconciling two processes otherwise difficult to explain. First, the two levels of stem cell proposed by Bjerknes and Cheng (1981) who suggested that the actual stem cells are scattered amongst the Paneth cells (the intercalated and/or crypt base columnar cells). These produce daughters that are displaced to the stem cell annulus at about cell position 4 and from these stem cells differentiation occurs. Such a model has implications in terms of the symmetry of division and p_{sm} but could be accommodated within the model in Fig. 6 with the crypt base stem cells being the actual stem cells and the cell position 4 stem cells being those T_1 cells with some selfmaintenance in the spiral.

The second point that can be accommodated is the observation that estimates for the number of clonogenic cells per crypt can vary depending on the dose of radiation being used to determine these numbers. Hendry (unpublished) has performed a detailed dose–response experiment where the results clearly show that, using small doses, the estimate for the number of stem cells per crypt is small (about 5 per crypt – similar to the estimate obtained using drugs, (Moore and Broadbent, 1980) and the estimate obtained using promethium, (Hendry *et al.* 1989) while high doses give numbers compatible with our earlier estimates of about 30 clonogenic cells per crypt (Potten *et al.* 1987). This suggests that the more severe the injury (in terms of cell kill or damage to the cell environment) the more differentiated transit cells can be triggered to renew the stem cells and regenerate a crypt. This is accommodated by the scheme in Fig. 6 which would suggest that as the level of injury is increased more of the transit population (e.g. T_2 and T_3) are called into the stem cell compartment by renewal.

(b) *Carcinogenic transformation*

It has already been pointed out (Potten, 1984) that a subtle genetic change in the elements that control p_{sm} may be an important change in at least the initiation event in multistage carcinogenesis. A fixed genetic change in p_{sm} from 0.5 to 0.52 would, in a crypt with a

24 h cell cycle for the 16 actual stem cells, result in about 7 extra stem cells after 100 cell cycles (i.e. 100 days). Since each extra stem cell division would produce 15 transit progeny cells, assuming the same 4 transit generations, this would mean an overproduction of about 500 cells over the 100 cell cycles in this one crypt. By the end of the period, the crypt would have about 7 extra stem cells presumably accommodated by an expansion from 16 to 23 cells per crypt circumference and about 100 extra transit cells presumably accommodated by an extension in crypt column height (Potten, 1984). Thus the crypt would resemble a hyperplastic structure; a situation that may represent a high risk for further carcinogenic transformation. The process would continue in the altered crypt eventually resulting in a hyperplastic crypt with proliferative cells near to, or on, the base of the villus. This, in addition, would induce a higher incidence of crypt fission due to the increased number of stem cells, giving rise to a spreading of the clone to many neighbouring crypts thereby possibly inducing adenomatous structures.

One way in which such a change in p_{sm} can be achieved in stem cells is by a change (a reduction) in the number of the receptors for a putative differentiation-inducing factor or a change in the binding capacity or efficiency of such receptors making it slightly less likely that the critical threshold levels of receptor binding or intracellular second messengers are reached to trigger differentiation. Thus sometimes the differentiation would be triggered, but on average it would be slightly less often than in neighbouring unaffected stem cells; for example, once less in about every 16 stem cell cycles in the example given above. Each new mutated stem cell produced would itself carry the higher level of p_{sm}. Thus small changes in p_{sm} could result after a long time (a time that is compatible with the long latent period in chemical carcinogenesis experiments) in one, or a series of, hyperplastic crypts that merely require a second event (promotional event) that allows one, or more, of the many genetically altered (initiated) cells to become malignant and invasive. Thus a paradigm for carcinogenesis to be tested by further experiments is a series of changes in the sequence: mutated stem cell→growth→single crypt with a clone of mutated cells→enlarged (hyperplastic crypt)→irregular crypt fission→adenoma→altered cell adhesion→invasion→carcinoma.

(c) *Carcinogen target cells*

It is clear that genetic changes (mutations, carcinogenic initiation) in the bulk of the proliferative crypt cells will be very transient features. Most proliferative crypt cells are transit cells, which invariably move out of the crypt and along the villus to fall off into the lumen of the gut three to five days after their birth from a cell division in the crypt (see Fig. 7). The only cells in which genetic changes can persist for times equivalent to the carcinogenic latent period (several months in the mouse and decades in man) are the stem cells, which are the only permanent residents of the crypt. The stem cells, are thus likely to be the most important carcinogenesis target cells. Regional differences in cancer incidence and susceptibility to specific carcinogens may therefore be related in part to the number, location and carcinogen sensitivity of the stem cells (see Potten, 1984).

Carcinogenic transformation may of course occur frequently in the proliferating transit cells but these altered cells will probably be shed from the villus within a few days, unless the single mutational carcinogenic change (initiation) affects simultaneously both proliferation (e.g. selfmaintenance probability) and cell-to-cell and/or cell-to-basement membrane attachment or cell migration ability. This seems improbable since these processes are unlikely to be genetically linked and physically it would be difficult for a cell to stop movement when totally surrounded by actively moving cells (it is like an individual trying to stop moving on a packed moving staircase). If such an arrest of movement occurred it would undoubtedly result in a considerable local disruption to cellular organisation and there is no evidence to support such a local micro-disruption to crypt architecture but it may in fact be difficult to detect. Carcinogenic transformation (initiations and/or promotions) can only be preserved in transit cells if such transformed cells could be encouraged to grow *in vitro*, which at present is unlikely for intestinal cells.

(d) *Stem cell division capacity and immortality*

The question of the extent of the division potential of small intestinal stem cells is clearly linked to the question of how many there are and how fast they cycle. Assuming that there are between 4 and 16 actual stem cells per crypt in the mouse with a cell cycle time of between 12 and 32 h then in the 3 year life of a laboratory mouse the stem cells would be expected to divide between 2200 and 820 times. For simplicity let us assume a 24 h cell cycle in which case about 1000 cell divisions would be expected in three years. In the wild state, the life expectancy of a mouse is about 6 months and it is on such a system that the evolutionary processes that determine stem cell function would have acted.

In 6 months the crypt stem cells would have divided about 180 times compared with the 1000 in the total life of a laboratory mouse. It is clear then that a wild mouse has intestinal stem cells that have a division capacity 6 times that which is needed for its 6 months expected life. These figures assume a constant cell production rate throughout life. These observations are further supported by those in other systems, for example in the bone marrow where it can be estimated that the stem cells divide about 200 times in the total life of the laboratory mouse but here serial transplantation experiments from old to young mice can be performed. Such transplanted bone marrow cells can then sustain the life of the preirradiated recipient mouse. This can be repeated up to 5 times. Thus bone marrow cells possess a division capacity up to 30 times that required for the

life span of a wild mouse (Potten and Lajtha, 1982; Potten, 1990).

In the human small intestine, the stem cell cycle time is unknown but the indications are that the average cell cycle time is between 4 and 8 times longer than that in the mouse. If the stem cells are cycling more slowly by the same amount, they would have a cycle of between 2 and 8 days, say 5 days. Thus in a 70 year life span the small intestinal crypt stem cells in man might divide 5000 times. Whether such a division capacity can be regarded as indicating immortality is debatable but the number of divisions that a normal stem cell is capable of is very large – 1000 in the murine small intestine and several thousands in man.

Transformed or established cell lines are commonly termed *immortal* yet few, if any, have been expanded through 1000 cell divisions which with a daily cell cycle or doubling would take 3 years. One of the oldest cell lines, HeLa was established in 1952 (Gey *et al.* 1952) and is currently commercially available 38 years later as the 30th to 100th passage in culture. The cells would probably have undergone 4–6 doublings per passage in their past history. This is equivalent to a total of up to 600 doublings since their establishment or the equivalent of up to about 2 years of continuous exponential growth at daily doublings. Some other cell lines may have undergone up to 500 passages i.e. could have been through 2000–3000 doublings. These figures are all of about the same order of magnitude of divisions that the normal intestinal stem cells undergo in the life of a mouse or man where they maintain their identity for this length of time, that is show no demonstrable genetic change (see Potten and Lajtha, 1982). Thus normal stem cells are capable of a large division potential and exhibit selfmaintenance over a period of up to at least about one thousand cell divisions.

Since there are about 7.5×10^5 crypts in the ileum of the mouse there must be between 3×10^6 and 1.2×10^7 stem cells in the ileum. Thus there are between 10^9 and 10^{10} stem cell divisions in the mouse small intestine in its 3 year life span. With a spontaneous mutation rate of approximately 1 in 10^6, about 10^3 or 10^4 spontaenous mutations would be expected, i.e. about 1–10 per day. It is therefore surprising that the small intestine rarely develops cancer. It must be very well protected by ill-understood mechanisms (see Cairns, 1975).

Each crypt in the intestine is believed to be clonal in its developmental origin (Ponder *et al.* 1985), although in reality the processes may be somewhat more complex than a simple expansion of the crypt from a single fetal progenitor cell (Schmidt *et al.* 1988). Thus during development a total of at least 7.5×10^5 crypt progenitor cells must be produced i.e. about 20 expansionary cell divisions. Thus a minimum of 20 doublings plus 2–4 for the expansion from one stem cell per juvenile crypt to 4–16 per adult crypt occurs in embryogenesis. This is a minimum number since it does not allow for the production of any differentiated or transit cells or for cell death. These may add approximately a further 40 % to the numbers above to give a total of about 30 doublings during development.

(G) Conclusions

The concept of stem cells has been defined and provides various parameters that can be applied for the identification of these cells. Ideally, several of these parameters simultaneously should be observed: however, this is rarely possible in practice. The nature and function of the stem cells inevitably makes them difficult to identify and study. Various aspects of their characteristics are influenced by the compartment size being analysed and the ultimate test for stem cell property, the clonal regeneration process, results in changing the identity of the cell leading to uncertainties in its identification and characterisation. These points have been discussed and a new model has been proposed in which a flexible transit population exists. Differentiation and maturation can occur within and from this transit population. The transit cells possess certain characteristics in common with stem cells and some in common with mature functional cells. i.e. are truely intermediate. It is suggested that the number of transit divisions is related to the range of differentiation options, i.e. pluripotency.

Each crypt in the small intestine must have at least one cell that has the property of stemness for the life of the animal and upon which all other cells in the crypt depend i.e. an actual stem cell. It is more likely that the crypt contains many actual stem cells, for example 4–16. In order to reconcile the data on the macroscopic behaviour of the crypts with these numbers, one has to conclude a predominantly asymmetric division pattern. However, an element of stochastic symmetric division is also necessary. The stem cells are probably passing through the cell cycle more slowly than the transit cells. The division potential of these cells is large, being at least 1000 in the life of a laboratory mouse. We have suggested elsewhere (Potten *et al.* 1990; Potten, 1990) that the cells are controlled by local interactive signals and can readily detect changes in their numbers and respond rapidly by changes in their cell cycle duration and their selfmaintenance probability. The cells can be mutated by chemicals or radiation but rarely develop into cancers even though there might be 10^9 or 10^{10} stem cell divisions in the life of a mouse. The large division potential makes these cells efficient tissue regenerators through colonal growth. The process of crypt regeneration from potential stem cells may begin very early after irradiation exposure (Potten *et al.* 1990, 1990). The precise number of cells that can regenerate the crypt and the mucosa, the potential clonogenic cells, is uncertain but is likely to be no more than about 30–40 per crypt, i.e. a double ring of cells at cell positions 4 and 5 from the bottom of the crypt. A double ring would suggest that the actual stem cells and their immediate daughters both possess stem cell attributes and can have their p_{sm} values changed to values above 0.5. Thus the clonogenic analyses suggest that the first transit population must retain some aspect of stemness. Within the transit population the selfmaintenance probability may be in principle progressively declining.

We have presented a new model for the crypt

hierarchy, a cork-screw or spiral model, which suggests that transit cells are truly an intermediate cell type between the stem cells and the mature cells and that they possess some characteristics of both stem and mature cells and have a certain flexibility to maintain the former or proceed to the latter. A number of questions remain, which should be the focus of research activity over the future decade. These include an analysis of the controls on the entire hierarchical system and the identification of specific differentiation markers particularly for the T compartment in systems like intestine; a more detailed investigation into the process of differention whereby actual stem cells become T cells and if present, of renewal, whereby T cells become actual stem cells, and the long term implications and consequences of this process; a broader analysis of the true pluripotency of stem cells at all stages from embryogenesis to tissues in adult animals (for example, are bone marrow stem cells really different from intestinal stem cells, and how different are they from embryonic stem cells?). What is the mechanism that determines the number of transit divisions? (is it an internal counting mechanism, clock, or is this governed by internal or external factors?) The haemopoietic system clearly shows that the number of divisions in this compartment is flexible. To what extent are proliferation and mature functional specialisation mutually exclusive? And finally what changes occur in the hierarchy during pathological abnormalities and during the carcinogenic transformation process.

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